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Inactivation of Tryptic Activity by a Human-Derived Strain of *Bacteroides distasonis* in the Large Intestines of Gnotobiotic Rats and Mice

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Tryptic activity disappeared and trypsin was no longer detected with an antitrypsin antiserum in the large intestines of gnotobiotic rats and mice monoassociated with a human-derived strain of *Bacteroides distasonis*, whereas tryptic activity was not modified in the small intestines. This function was shown to be strain specific.

Trypsin, one of the main pancreatic enzymes, plays a major role in proteolysis in the small intestine but disappears in the large intestine where a complex microbial flora is present. Indeed, feces of axenic rodents always exhibit a high level of fecal tryptic activity, whereas those of their conventional counterparts exhibit little or no fecal tryptic activity (4, 9, 10). It is likely that the human flora of the large intestine is involved in the disappearance of tryptic activity, since according to Macfarlane et al. (5) levels of protease activities are lower in the feces than in the human ileal effluent. In addition, Bohe et al. (1) observed an increase in pancreatic proteases in the feces of patients treated with antibiotics. Bacterial strains able to inactivate tryptic activity do not appear very early in the large intestines of infants. Norin et al. (8) reported that fecal tryptic activity is still present in 20-month-old infants, whereas it is no longer detected in 46- to 61-month-old children. Trypsin inactivating activity can disappear under some pathological conditions such as diagnosed Crohn's disease (11, 13).

The identity of bacterial strains responsible for the inactivation of fecal tryptic activity is still unknown. Such bacteria were shown to appear very rapidly in axenic rats associated with a conventional rat flora (7), but they have not been isolated yet. No inactivation of the fecal tryptic activity appears in gnotobiotic mice inoculated with both a *Bifidobacterium* strain and a *Lactobacillus* strain (12). The aim of our work was to determine the bacterial strains isolated from the predominant human fecal flora which inactivate the tryptic activity.

Assays for tryptic activity were performed as described by Midtvedt et al. (7), using the synthetic substrate Nα-benzoyl-L-arginine *p*-nitroanilide hydrochloride (Sigma Chemical Co., St. Louis, Mo.). Samples of feces and intestinal contents from one human and rodents were thoroughly mixed with saline (1:5), placed at 4°C for 2 h, and centrifuged (35,000 × *g*, 4°C, 30 min), and supernatants were kept at −20°C before the assays were performed. Bovine pancreas trypsin type XIII, L-1-tosylamide-2-phenylethylchloromethylketone (TPCK) treated, (Sigma) was used for the construction of a standard curve. All samples and standards were analyzed in parallel. Tryptic activity was expressed in micrograms of trypsin per gram of fresh sample, the threshold being 30 μg/g. Western blotting (immunoblotting) with antitrypsin antiserum was done on supernatants of nondiluted cecal contents as follows. Proteins from supernatants (5 μl) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were transferred onto nitrocellulose filters. The filters were quenched by using 4% fat-free dry milk powder and 1 M NaCl in PBS (PBS is 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). The antiserum directed to trypsin was diluted 1:3,000 with a suspension of 3% fat-free dry milk powder in a solution of 1 M NaCl in PBS and applied to the nitrocellulose filters for 2 h at room temperature. After washing, peroxidase-labeled immunoglobulins (Immunotec, Marseille, France) directed to the Fc fragment of the bound antibody were used to detect the antigen. Diaminobenzidin (35 mg) and 50 μl of H_2O_2 solubilized in 100 ml of PBS were used as substrates. The reaction was stopped by rinsing the filters in distilled water. Protease activity was determined as previously described (2) in samples supplemented or not with 1 mg of trypsin inhibitor (Soybean Type-1-S; Sigma) per ml; the protease activity titer was defined as the quantity of pig immunoglobulin G bound to microtiter plates which was hydrolyzed by the samples within 2 h at 37°C. Axenic C3H/He adult mice and Fischer 344 adult rats were reared in Trexler-type isolators and fed ad libitum with a commercial diet (RO340; UAR, Villemoisson, France) sterilized by gamma irradiation (40 kGy). They were inocu-

TABLE 1. Comparative evolution of fecal tryptic activity in axenic rats and rats monoassociated with *B. distasonis* D4 or E9

Days after inoculation of bacterial strains	Fecal tryptic activity[a] in:		
	Axenic rats	Rats monoassociated with:	
		Strain D4	Strain E9[b]
0	682 ± 43	675 ± 69	551 ± 77
4	747 ± 80	782 ± 31	738 ± 18
10	724 ± 24	686 ± 27	200^{α} ± 73
13	612 ± 124	832 ± 78	51^{β} ± 13
24	736 ± 182	785 ± 46	35^{β} ± 5

[a] Expressed in micrograms of trypsin per gram of fresh feces; means ± standard errors of the mean (SEM) for four rats individually sampled.

[b] Data with superscript letters are significantly different from those on day 0: α, $P < 0.05$; β, $P < 0.001$.

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TABLE 2. Tryptic activity in the first, second, and third parts of the small intestines (Si1, Si2, and Si3, respectively), ceca and colons of axenic rodents and of rodents monoassociated with *B. distasonis* D4 or E9

Bacterial status[a]	Hosts	Tryptic activity[b] in:				
		Si1	Si2	Si3	Cecum	Colon
Axenic	Rats	282 ± 34	713 ± 138	1,783 ± 110	639 ± 135	505 ± 34
	Mice	1,326 ± 350	1,207 ± 211	1,337 ± 127	1,159 ± 78	1,182 ± 90
Monoassociated with strain D4	Rats	248 ± 41	1,641 ± 49	2,508 ± 151	689 ± 14	765 ± 80
	Mice	965 ± 498	1,099 ± 78	1,323 ± 164	845 ± 156	835 ± 131
Monoassociated with strain E9	Rats	309 ± 59	1,266 ± 157	1,576 ± 124	38 ± 4	<30
	Mice	1,242 ± 300	1,625 ± 207	1,458 ± 92	<30	<30

[a] Monoassociated rats and mice were sacrificed 25 days after bacterial association.
[b] Expressed in micrograms of trypsin per gram of fresh contents; means ± SEM for four rats and four mice individually sampled. All data in ceca and colons of rats and mice monoassociated with strain E9 are significantly different from those in ceca and colons of axenic rodents ($P < 0.001$).

lated through the orogastric route with 1 ml of the bacterial inoculum. Inocula were either a 24-h culture at 37°C in liquid brain heart infusion (BHI) medium (Difco Laboratories, Detroit, Mich.) or complex bacterial suspensions prepared from culture on agar BHI medium. All cultures were done in an anaerobic chamber. The complex bacterial suspension from human feces was prepared by diluting 10-fold the feces in liquid BHI medium and plating 0.1 ml of the 10^{-7} dilution on agar BHI medium in the anaerobic chamber. The plate was incubated for 5 days at 37°C and then washed out with 2 ml of saline, and the suspension was inoculated in axenic recipient mice. For analysis, all samples of feces and intestinal contents from the rodents were collected early in the morning. Bacterial counts were done by plating 0.1 ml of adequate dilutions of intestinal contents on agar BHI medium in the anaerobic chamber; incubation was for 3 days at 37°C. Student's *t* test was used to compare mean values of trypsin concentrations and of $\log_{10}$ bacterial counts.

Feces from a human volunteer, exhibiting less than 30 μg of trypsin per g, were inoculated in axenic recipient mice as previously described. Trypsin activity dropped from 697 μg/g before inoculation to <30 μg/g 14 days later in the mouse feces (data not shown). Feces of previous recipient mice were 10-fold diluted in the anaerobic chamber, and 100 colonies were isolated from the highest dilution plated on the agar BHI medium. As Macfarlane et al. demonstrated that several *Bacteroides* strains are proteolytic (5) and that *Bacteroides fragilis* strains are able to utilize trypsin in vitro (6), we decided to screen among the 100 colonies all the *Bacteroides* strains and to inoculate other axenic mice with this mixed *Bacteroides* suspension. Fecal trypsin activity also dropped to <30 μg/g of mouse feces 14 days later. Then, five *Bacteroides* strains, which differed by their morphology in BHI medium, were inoculated alone or together in other axenic mice. Only one of them, strain E9, decreased significantly fecal tryptic activity in monoassociated rats from days 10 and 13 after inoculation (Table 1). Fecal tryptic activity in mice monoassociated with strain E9 also dropped to <30 μg/g from day 13 after inoculation (data not shown). This strain was identified as *Bacteroides distasonis* by using the API system (API, La Balme Les Grottes, France) and monoclonal antibodies (3). Another strain of *B. distasonis*, strain D4, isolated from the same recipient mice and screened by the same techniques, did not significantly modulate tryptic activity within 24 days in monoassociated rats (Table 1). Table 2 shows that trypsin inactivation was only achieved in the ceca and colons of rats and mice monoassociated with strain E9. Tryptic activity in the last part of the small intestines (Si3) was not significantly different in rodents monoassociated with strain E9 compared with that for the axenic rodents. This result was correlated with levels of strain E9 populations which were much lower in the stomach and small intestine than in the cecum (Table 3). Table 4 shows that strain E9 elicited a protease activity in the large intestines of monoassociated mice, since trypsin inhibitor was not able to totally inactivate protease activity of their cecal contents like in those of axenic mice. Figure 1 shows that trypsin was no longer detected with the antitrypsin antiserum in those ceca of mice monoassociated with strain E9 where tryptic activity had disappeared, whereas trypsin elicited a strong reaction with the antibody in the ceca of axenic mice. This strongly suggests that strain E9 degrades trypsin in the ceca of monoassociated mice. However, degradation products were not evidenced by using the polyclonal antibody. One may suppose either that such products were utilized by the host tissues or by the bacterial cells or that they cannot be detected by the antibody because of the disappearance of the trypsin epitopes.

Our results show for the first time that a *B. distasonis* strain is able to inactivate residual tryptic activity in the large intes-

TABLE 3. Population levels of *B. distasonis* D4 and E9 in the first, second, and third parts of the small intestines (Si1, Si2, and Si3, respectively), stomachs, and ceca of monoassociated rats

Gut segment	$\log_{10}$ counts (mean ± SEM, $n = 4$) in rats monoassociated with[a]:	
	Strain D4	Strain E9
Stomach	4.6 ± 0.1	5.8 ± 1.1
Si1	5.2 ± 0.4	5.1 ± 1.0
Si2	4.4 ± 0.7	5.3 ± 0.8
Si3	5.7 ± 0.1	7.8 ± 1.0
Cecum	10.2 ± 0.1*	10.4 ± 0.0*

[a] *, significantly different from data obtained with Si3 ($P < 0.001$). Monoassociated rats were sacrificed 25 days after association with strain E9.

TABLE 4. Proteolytic activity on pig IgG[a] in cecum contents of axenic mice and mice monoassociated with *B. distasonis* E9

Mouse bacterial status	μg of pig IgG hydrolyzed/g of cecal contents[b]	
	With trypsin inhibitor	Without trypsin inhibitor
Axenic	<0.8	6,309 ± 0.5
Monoassociated with strain E9	63 ± 0.5	398 ± 0.5

[a] IgG, immunoglobulin G.
[b] Means ± SEM for six mice. Monoassociated mice were sacrificed 25 days after association with strain E9.

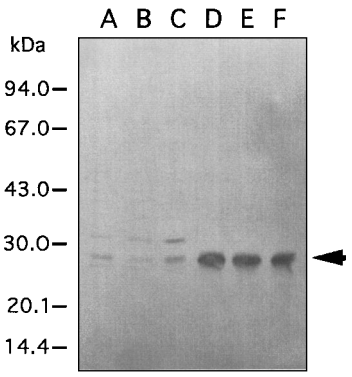

FIG. 1. Trypsin detection by Western immunoblotting with an antitrypsin antiserum in the nondiluted cecal supernatants of axenic mice and mice monoassociated with strain E9 for 25 days. Lanes A, B, and C, supernatants of three mice monoassociated with strain E9. Lanes D, E, and F, supernatants of three axenic mice. Molecular masses are indicated on the left.

tines of monoassociated rodents. This function appears to be strain specific, since another closely related *B. distasonis* strain had no effect on tryptic activity in vivo. As strain E9 was present in the predominant fecal flora of the human donor, it is very likely that trypsin inactivation can be achieved in the large intestine by the *B. distasonis* strain we have isolated. It would be of interest to correlate such a bacterial function with the establishment of *B. distasonis* strains in the intestines of infants. Otherwise, it has to be known whether other strains from the predominant flora of the human large intestine may exert the same function as *B. distasonis* E9.

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